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SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF CELLULOSE AND WHEAT STRAW TO ETHANOL: EVALUATION OF THERMOTOLERANT YEAST AND ß-GLUCOSIDASE SUPPLEMENTATION

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ABSTRACT

From preliminary glucose and simultaneous saccharification and fermentation (SSF) screening tests of 10 promising microbial strains at temperatures ranging (37 to 47°C), five yeast, Saccharomyces cerevisiae, S. uvarum, Candida lusitaniae, C. brassicae, and Brettanomyces clausenii, were selected as good fermenters in pure or mixed culture for more detailed evaluations. The parameters measured included ethanol concentration, yeast cell density, residual sugars, and cellulose concentration. Mixed cultures of S. cerevisiae with B. clausenii and C. lusitaniae with S. uvarum fermented best on Sigmacell-50 cellulose substrate, apparently because of rapid cellobiose removal. However, for pretreated wheat straw, SSF with a single culture of a strong glucose-fermenting ethanol-tolerant yeast achieved higher conversion rates and yields than the mixed culture if the cellulase enzyme was supplemented with ß-glucosidase. Enzyme supplementation alleviates possible problems of mixed culture maintenance, which may arise in continuous fermentations.

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INTRODUCTION

The simultaneous saccharification and fermentation process has been researched as a method of converting lignocellulosics to ethnol since the 1977 publication by Takagi et al (17). Ethanol can be used as a neat fuel in internal combustion engines or blended with gasoline as an octane enhancer and fuel extender. To make ethanol from the SSF process competitive with the price of petroleum, factors such as yeast viability, enzyme cost, rate of hydrolysis, and ethanol yield need to be improved.

Microbes selected for this paper have been reported in many publications concerning cellulose, glucose, cellobiose, or xylose fermentations at various temperatures. SSF studies were done by Blotkamp et al (3) with Saccharomyces cerevisiae, S. uvarum, and Candida brassicae with the cellulase enzyme from T. reesei to observe the effects of different yeasts, cellulase loadings, and substrate concentrations at 40 and 45°C. S. uvarum was also evaluated in an SSF study with the cellulase from Penicillium funicolsum (4). Candida Acidothermophilum (5), C. brassicae (1,2,3,9,12,14,17), C. lusitaniae (6,7,8), and Schizosaccharomyces pombe (17) have all been reported for production of ethanol. Brettanomyces clausenii and a S. cerevisiae mutant strain (D_5A) have been used in three of our recent publications on SSF (8,16,20). Finally, the bacteria screened in this work were two strains of Zymomonas mobilis based on results comparable to S. cerevisiae (13,15,19).

This paper summarizes three interrelated projects: screening of 10 microbes for SSF, a more complete evaluation of the yeasts selected from the screenings, and a preliminary study involving the application of the best of these yeast to pretreated wheat straw. We also looked at supplementing of SSFs with ß-glucosidase. These studies were performed to evaluate all the microbes reported in the literature as good ethanol producers under the same temperatures, enzyme loadings, substrate loadings, and substrate and enzyme types to get a clearer picture of their potential for SSF. Another factor considered in the selection was the thermotolerance of the microbes since the rate-limiting saccharification step would increase with increases in temperatures up to 45 to 50°C, all other factors being equal. Once yeast were selected from initial screening tests, more complete data were gathered from focused SSFs. Sigmacell-50 substrate was initially used in the screening tests and fermentations from which we gathered more complete data, but to get a more realistic picture of SSF potential, wheat straw was employed as the substrate for the best performers.

MATERIALS AND METHODS

Materials

Strains and their numbers ordered from the American Type Culture Collection (ATCC), Rockville, Maryland, are as follows: Candida acidothermophilum 20831, Candida brassicae 32196, Saccharomyces cerevisiae 4126 and 4132, Saccharomyces uvarum 26602, and Zymomonas mobilis 10988 and B4490. Strains ordered from the Northern Regional Research Laboratories (NRRL), U.S.D.A., Peoria, Illinois, were: Schizosaccharomyces pombe 1358, Candida lusitaniae 5394, and Brettanomyces clausenii Y 1414. S. cerevisiae D_5A is a SERI strain genetically derived from Red Star baker's yeast. Growth and fermentation yeast peptone media came from Difco, and the sugar for starting up inoculations came from Sigma Chemical Company. Sigmacell-50 substrate was ordered from Sigma. The cellulase used was Genencor 150L from Genencor Inc., San Francisco, California. Two separate batches of this enzyme were employed in these studies. ß-Glucosidase (Novozyme-188) came from NOVO Laboratories, Inc., Wilton Connecticut. Fermentation vessels are 6-L Braun Biostat V fermenters for large-scale, more comprehensive studies from B. Braun Instruments, Burlingame, California, or 50 and 250 mL Pyrex graduated flasks for the screening tests.

Methods

Preliminary glucose screening fermentations (25 mL) and small-scale SSFs (100 mL) were run in 50 and 250 mL flasks, respectively, constructed to vent CO_2 into water traps. All fermentations used 1% yeast extract and 2% peptone as media and were agitated at 150 RPM. The substrate for the SSFs was Sigmacell-50 or wheat straw at 7.5, 10, or 26% (w/v) cellulose concentrations. The Genencor enzyme was at 7, 13, and 26 IU/g substrate, in which IU represents an international unit of filter paper activity in micromoles of glucose per minute (10). A lipid mixture of 5 mg/L ergosterol and 30 mg/L oleic acid plus penicillin and streptomycin at 10 mg/L were added to SSF media to improve ethanol tolerance and to minimize contamination, respectively. The media, substrate, and lipids were autoclaved in the vessel before the addition of antibiotics and inocula with exception of the wheat straw SSFs, where all previously sterilized liquid components were mixed together and added to autoclaved wheat straw to assure better mixing of enzyme.

Wheat straw (12.5 kg) was pretreated with dilute sulfuric acid [0.5% (v/v)] in a Pfaudler batch reactor with 137 L of distilled water. The pH of the reactor was 1.54 and temperature was held at 140°C for 1 hour with stirring. The pretreated straw was then centrifuged and washed several times, and the pH was brought up to between 4.0 and 4.5 with 70 wt% NaOH.

Ethanol concentrations were measured in the supernatant via gas chromatography on a Hewlett Packard 5880 A, Porapak Q80/100 column. The internal standard was 4% isopropanol. Residual sugars (glucose and cellobiose) were determined as glucose by incubation of the sample 2 mg/mL with almond extract cellobiase from Sigma for 1 hour at 37°C, and total sugars were measured on the glucose analyzer from Yellow Springs Instruments. Cell density was measured as colony forming units (CFU) by platting serial dilution on YPD or YPC plates. Cellulose concentration was measured as follows: the yeast cells were prehydrolyzed with dilute hydrochloric acid 3% (v/v) at 80°C for 1 hour.

The residue was washed and centrifuged several times to recover solids. Then the yeast and other components were selectively dissolved in 2.5% (w/v) sodium hydroxide by heating at 65°C for 30 minutes. This step was followed by several more washes and centrifugations before recovering the cellulose on a pre-weighed Millipore 0.45 m HA type filter that is dried at 45°C overnight before weighing.

Results reported in percent equivalent conversion of cellulose represent the percent of the feed cellulose that is required to provide the measured ethanol concentration assuming a 90% yeast efficiency; the remaining 10% of the sugars are assumed to be used for cell growth and maintenance. This measure gives us an indication of the consumption of substrate from only the ethanol concentrations measured in the screening experiments, and puts all screening tests on the same basis for comparison with the straight saccharification of cellulase to sugars. For the larger scale SSFs, actual cellulose conversion is measured to check the calculated value.

RESULTS

Throughout our work, we used Genencor 150L cellulase since it performed the best of all enzymes considered in a brief screening test (16). Two batches of Genencor cellulase with differing levels of activities were used for the fermentations reported here. Table 1 lists characteristics of the Genencor 150L enzyme. The important activity to note is the ß-glucosidase level in these enzyme preparations: batch II exhibits about a 30% decrease in ß-glucosidase activity relative to batch I which slows the cellobiose to glucose conversion rates. Also, the total filter paper units decrease from 105 to 85 IU/mL. An interesting point brought out by the IUPAC 1987 revision of 'Measurements of Cellulase Activities' is that the level of ß-glucosidase in an enzyme preparation may affect the results of the cellulase assay, particularly for the filter paper units (10).

Our original supply of Genencor cellulase (batch I) was exhausted on the preliminary small-scale screenings, and the following work used the less active (batch II) cellulase for saccharification. Switching cellulase batches resulted in about a 20% decrease in ethanol production. Nevertheless, batch II was still valuable in comparing the yeast characteristics on a consistent basis for large-scale SSFs, and, if supplemented with ß-glucosidase for saccharification of Sigmacell-50, its total activity increases substantially (Figure 1).

Based on the literature, 10 strains of microorganisms were chosen for the small scale screening: Candida brassicae, C. lusitaniae, C. acidothermophilum, Saccharomyces cerevisiae, S. uvarum, Schizosaccharomyces pombe, and Zymomonas mobilis. Glucose fermentations were run on these strains at 37, 41, 45 and 47°C for seven days to test their thermotolerance (16). As a result of this screening, three of the eight yeast (S. cerevisiae 4126 and 4132 and S. pombe 1356) and the two bacterial strains of Zymomonas were eliminated because of poor performance at 41°C and above. Brettanomyces clausenii was included as a control because even though it will not grow over 37°C, it gave the best results in our previous SSF studies.

The five remaining yeast were run in screening SSFs with 7.5, 10 and 15%

Sigmacell-50 cellulose and cellulase (batch I) concentrations of 7, 13 and 26 IU/g of substrate, resulting in nine fermentations for each yeast at each temperature. Results of this SSF screening are presented in Table 2 as final percent equivalent cellulose conversions on Sigmacell-50. Some of these yeast were run in mixed cultures to evaluate the cellobiose fermenting yeast B. clausenii and C. lusitaniae with the strong glucose fermenters S. cerevisiae, S. uvarum and C. brassicae. The high limit temperature for this study was chosen as 45°C since previous publications report growth at this temperature and because the cellulase enzyme shows optimal hydrolysis at 45°C (16).

As Table 2 demonstrates, mixed culture I of B. clausenii and S. cerevisiae achieved higher equivalent cellulose conversions than any other yeasts at any enzyme/substrate loading at 37°C with the exception of 26 IU/g for 10 and 15% substrate. At this loading, C. brassicae and S. cerevisiae were competitive with mixed culture I, and C. lusitaniae excelled for the 10% substrate. For all the temperatures, mixed culture I does the best in final yield with the exception of mixed culture II which looks better at an enzyme loading of 13 IU/g for 10% substrate loading at 41°C.

Figure 1 illustrates the final percent equivalent conversions for the four most thermotolerant at selected temperatures and substrate concentrations. S. uvarum shows the most consistent results for temperatures of 37, 41, and 43°C, while the three Candida strains, lusitaniae, brassicae, and acidothermophilum, demonstrate a decrease in percent conversion with increase in temperature and substrate. All the yeast did poorly at 45°C.

To present a rate comparison of the yeast, Figure 2 reveals the two- and four-day and final percent conversions of the four thermotolerant yeast at 10% substrate with 13 IU/g enzyme loading at selected temperatures. C. lusitaniae has the fastest two- and four-day fermentations at 37 and 41°C, whereas the other three yeast give comparable rates of conversion.

The mixed culture rate data at 10% substrate and 13 IU/g enzyme loadings were compiled in Figure 3 to demonstrate some close similarities among them. Here, we see that mixed culture I (B. clausenii and S. cerevisiae) does best at 37°C and mixed culture II (C. lusitaniae and S. uvarum) is best at 41°C. Both mixed cultures II and III (C. lusitaniae and C. brassicae) show good rates for the higher temperatures and tend to complete the fermentation one or two days ahead of mixed culture I.

S. uvarum, C. lusitaniae, C. brassicae, and mixed cultures I and II were selected for the more detailed large-scale SSF evaluation. These particular yeast were chosen from the screening tests because they performed best, and we wanted to obtain more complete data on their performance. Figure 4 demonstrates the average residual sugars of the yeast in large scale at selected temperatures. Mixed culture I keeps the sugars at the lowest levels, with mixed culture II and S. uvarum close behind. The Candida species, on the other hand, show more build up in residual sugars, which are known to inhibit the cellulase activity (18).

The cell density is high for C. brassicae and S. uvarum at 37°C but decreases with increase in temperature. C. lusitaniae tends to die early in these SSFs because of low ethanol tolerance, yet we see good viability for this yeast when combined with S. uvarum in mixed culture II. Both mixed

cultures have high cell densities because of their compatibility and double inoculum at the start of the fermentations (Figure 5).

Figure 6 illustrates the cellulose hydrolysed in seven days for all the large-scale SSFs. Mixed culture II at 41°C is the best performer based on this parameter. In Figure 7, the two- and four-day and final percent equivalent conversions of cellulase are presented for the large scale SSFs. C. lusitaniae has good initial rates, which decrease as the fermentation proceeds. C. brassicae and S. uvarum do well in comparison to the mixed cultures, especially at 37°C. Mixed culture II (41°C) performs the best for rates and final yields, with mixed culture I at 37°C a close second.

At this point, a preliminary evaluation of wheat straw was introduced to measure performance of the SSF process with actual pretreated substrates. Since the mixed cultures of cellobiose-fermenting yeast with ethanol-tolerant strains gave better results than for either yeast alone, reduction in the strong enzyme inhibitor cellobiose appears to be desirable, and supplementation with ß-glucosidase appeared a promising alternative approach. Therefore, we also compared yeast supplemented with ß-glucosidase to our most promising yeast and mixed cultures.

A question about the ß-glucosidase activity of the Novo-188 cellobiase enzyme used in supplementation for SSFs was clarified by running PnPGU assays at selected temperatures of 37, 40 and 50°C. It was found that at 37°C, we get 125 IU/mL, at 40°C the activity is 200 IU/ml, and at 50°C, it increases to around 500 IU/ml. These data were important because the wheat straw SSFs were being run at 37°C, so the ratios of cellobiase to cellulase activities had to be determined with this in mind.

A straight saccharification was run at 45°C on wheat straw with and without ß-glucosidase supplementation at ratios (based on 37°C assay) of 1:1, 1:4, and 1:8 IU batch II Genencor cellulase to IU Novo-188 cellobiase. These were run at 7, 13, and 26 IU Genencor cellulase/7.5% glucan in straw. Complete saccharification was achieved in five days with 1:8 cellobiase supplementation at 26 IU Genencor. In wheat straw SSFs, we see complete equivalent cellulose conversions at 1:4 and 1:8 in five days, showing faster saccharification of substrate in SSF at 37°C than with straight saccharification at 45°C (Figure 8).

Small-scale SSFs were run with S. cerevisiae (D_5A) and S. uvarum with and without ß-glucosidase (Novo-188) supplementation. These yeast were run at 7, 13, and 26 IU (batch II) Genencor cellulase/gram of (7.5%) glucan substrate in straw. The ß-glucosidase was supplemented at ratios of 1:1, 1:4, and 1:8 in IU cellulase to IU ß-glucosidase. SSFs were run at 37°C, so the activity of the cellobiase supplementation reflects this temperature. Mixed culture I of B. clausenii and S. cerevisiae was run on wheat straw without the supplementation as a control. Results show an improvement in SSF with the straw substrate and with cellobiase supplementation. In fact, the supplemented SSFs gave better results in rate and yield than the previous runs on batch I Genencor, which had 20% more ß-glucosidase activity. There is a 15% increase in cellulose conversion for wheat straw with batch II enzyme versus SSFs with batch I enzyme with Sigmacell-50 and for cellobiase supplementation versus no supplementation with batch II enzyme in the wheat straw SSFs. Saccharification rates improved by two days for the wheat straw SSFs and straight sac-

charification versus Sigmacell-50 (Figure 8).

DISCUSSION

In the small-scale screening experiments, mixed cultures of a cellobiose fermenter and a strong glucose-fermenting yeast reduced the residual sugars (cellobiose, in particular), increasing the rate of hydrolysis and product yield. Large-scale SSFs demonstrated that there is an increase in residual sugars with increasing temperature due to a decrease in yeast viability. We also found that ß-glucosidase supplementation greatly improves the fermentation rates when used with a strong glucose fermenting yeast by minimizing cellobiose accumulation. Thus, buildup of residual sugars appears to inhibit the enzyme performance more than can be compensated for by a temperature increase. Pretreated wheat straw performs better in the SSF process than the pure crystalline cellulose that was used before. Finally, a single culture of a strong glucose fermenter like S. uvarum appears to be a good choice when supplemented with ß-glucosidase enzyme.

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Table I

Characterization of Cellulases

	Genencor 150L Batch I	Genencor 150L Batch II
Dry wt (mg/mL)	164.	185.
Protein Units	127.	135.
% Protein	77.7	73.
CHO (mg/mL)	32.	44.
% CHO	19.	23.8
IU/mL	106.	84.7
PnPGU/mL	160.	111.
CMC/mL	2500.	4500.
IU/mg Protein	0.83	0.63
PnPGU/mg Protein	1.25	0.82
CMC/mg Protein	19.7	33.3

Nomenclature:

CHO - carbohydrate content as determined by phenol-sulphuric acid assay

IU - filter paper assay for saccharifying cellulase expressed in International Units

PnPGU - β-glucosidase assay with p-nitrophenyl-β-glucopyranoside substrate expressed in International Units

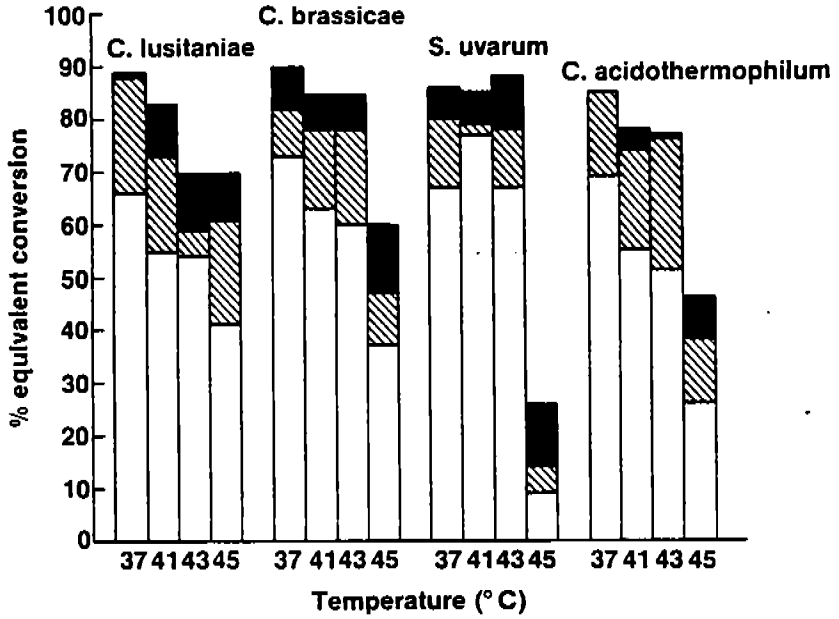
CMC - carboxymethyl cellulose assay for endo-β-1,4 glucanase expressed in International Units

Table 2. Performance of six yeast strains and their mixed cultures measured in percent equivalent cellulose conversions at end of runs for selected temperatures, substrate concentrations, and cellulase (Genencor 150L) loadings for small scale SSF runs with Sigmacell 50 cellulose

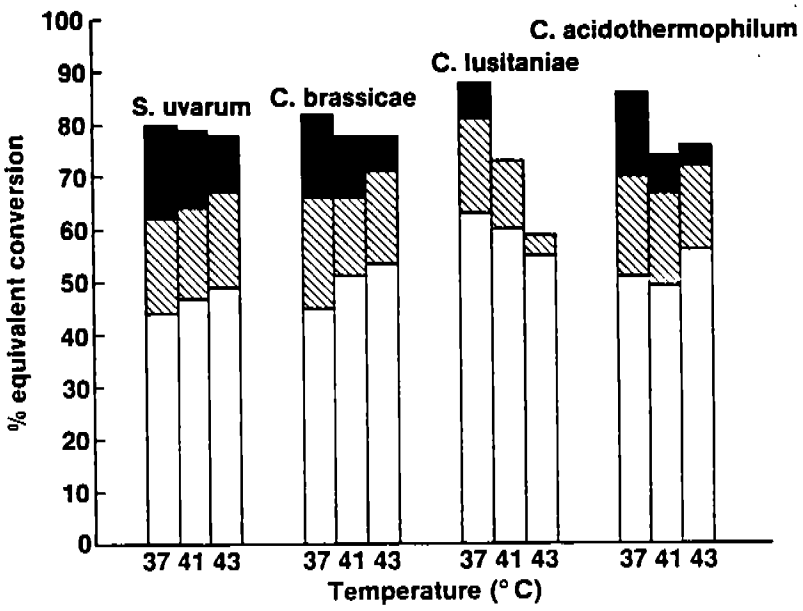
Substrate Concentration	7.5%					10%					15%			
Temperature	37°C	41°C	43°C	45°C		37°C	41°C	43°C	45°C		37°C	41°C	43°C	45°C
7 IU/g														
Mixed Cult. I	95	*	*	*	Mixed Cult. I	88	*	*	*	Mixed Cult. I	72	*	*	*
Mixed Cult. II	84	83	78	*	Mixed Cult.II	80	81	75	*	Mixed Cult.II	68	67	61	*
Mixed Cult. III	86	88	78	*	Mixed Cult.III	83	84	76	*	Mixed Cult.III	69	66	60	*
B.clausenii	86	*	*	*	B.clausenii	84	*	*	*	B.clausenii	72	*	*	*
C.lusitaniae	83	77	74	37	C.lusitaniae	80	75	60	56	C.brassicae	70	59	54	35
C.brassicae	83	81	84	43	C.brassicae	79	77	75	40	C.lusitaniae	65	55	48	43
S.cerevisiae	77	77	26		S.cerevisiae	73	77	29	*	S.uvarum	65	68	60	15
C.acidotherm.	74	76	61	42	S.uvarum	72	80	76	12	S.cerevisiae	64	66	36	*
S.uvarum	68	79	79	23	S.acidotherm.	67	59	53	37	C.acidotherm.	55	46	45	28
13 IU/g														
Mixed Cult. I	98	*	*	*	Mixed Cult. I	89	*	*	*	Mixed Cult. I	77	*	*	*
Mixed Cult. II	91	85	84	*	Mixed Cult.II	84	92	80	*	Mixed Cult.II	74	68	64	*
Mixed Cult. III	90	97	84	*	Mixed Cult.III	83	85	78	*	Mixed Cult.III	73	68	65	*
C.brassicae	90	84	84	60	C.lusitaniae	88	73	59	61	S.cerevisiae	76	64	37	*
C.lusitaniae	89	83	70	70	C.acidotherm.	86	74	76	38	B.clausenii	72	*	*	*
B.clausenii	89	*	*	*	B.clausenii	85	*	*	*	C.brassicae	73	63	60	37
S.uvarum	86	85	88	26	C.brassicae	82	78	78	47	C.acidotherm.	69	55	51	26
S.cerevisiae	87	87	41	*	S.cerevisiae	81	76	39	*	S.uvarum	67	77	67	9
C.acidotherm.	83	78	77	46	S.uvarum	80	79	78	14	C.lusitaniae	66	55	54	41
26 IU/g														
Mixed Cult. I	98	*	*	*	C.lusitaniae	96	74	64	58	C.brassicae	78	67	60	42
Mixed Cult. II	90	90	86	*	Mixed Cult. I	93	*	*	*	S.cerevisiae	79	67	38	*
Mixed Cult. III	91	93	89	*	Mixed Cult.II	82	84	80	*	Mixed Cult. I	78	*	*	*
C.lusitaniae	94	88	77	67	Mixed Cult. III	84	85	82	*	Mixed Cult. II	74	67	63	*
C.brassicae	91	89	86	66	C.brassicae	93	85	78	49	Mixed Cult. III	75	68	65	*
S.cerevisiae	91	86	55	*	C.acidotherm.	86	80	67	39	C.uvarum	77	78	63	27
B.clausenii	89	*	*	*	S.uvarum	86	88	80	16	C.acidotherm.	73	67	54	24
C.acidotherm.	89	89	88	48	S.cerevisiae	85	82	45	*	B.clausenii	72	*	*	*
S.uvarum	86	89	89	21	B.clausenii	83	*	*	*	C.lusitaniae	66	53	49	39

Mixed Culture I B. clausenii and S. cerevisiae
Mixed Culture II C. lusitaniae and S. uvarum
Mixed Culture III C. lusitaniae and C. brassicae

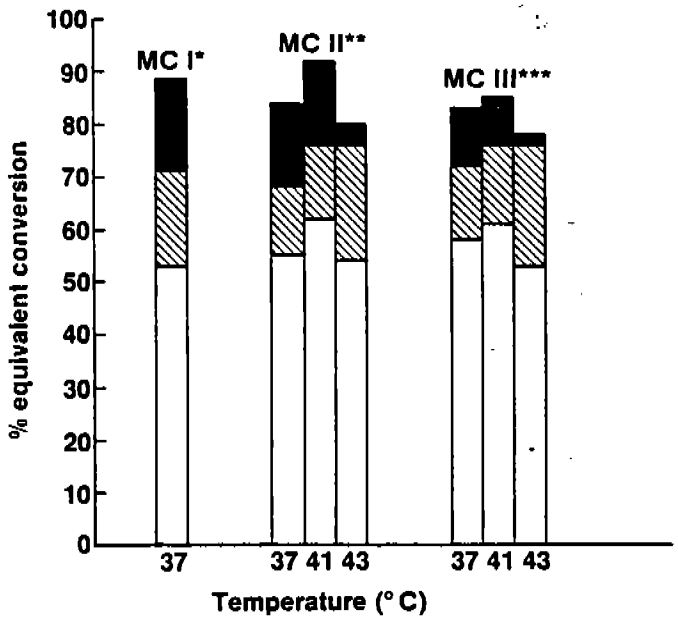
*Not run since yeast didn't ferment glucose at this temperature



1. Comparison of the four thermophilic yeasts percent equivalent conversions at selected temperatures and Genencor (batch I) cellulase loading of 13 IU/g Sigmacell-50 cellulose at 7.5% (■), 10% (▨) and 15% (□) w/v.

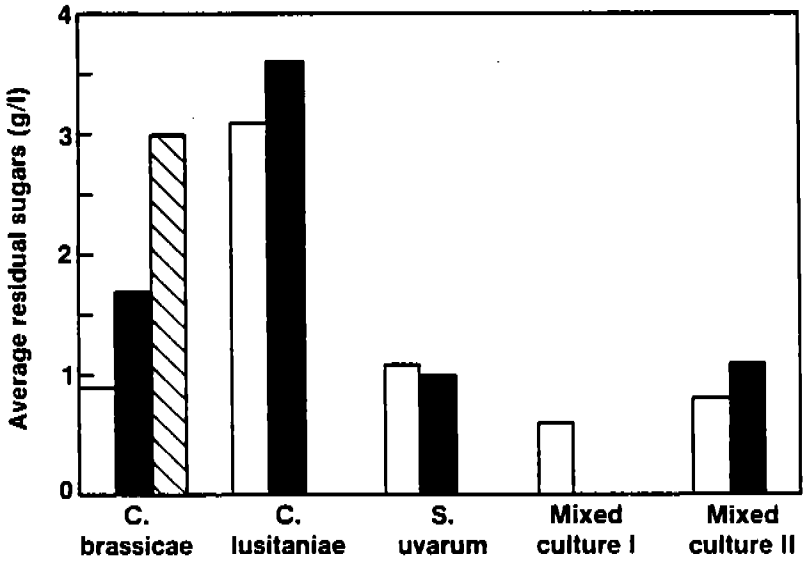


2. Comparison of the four thermophilic yeast at selected temperatures with 13 IU/g Genencor (batch I) cellulase enzyme loading and 10% Sigmacell-50 cellulose substrate concentrations for 2 days (□), 4 days (▨) and final day (■) percent equivalent conversions.

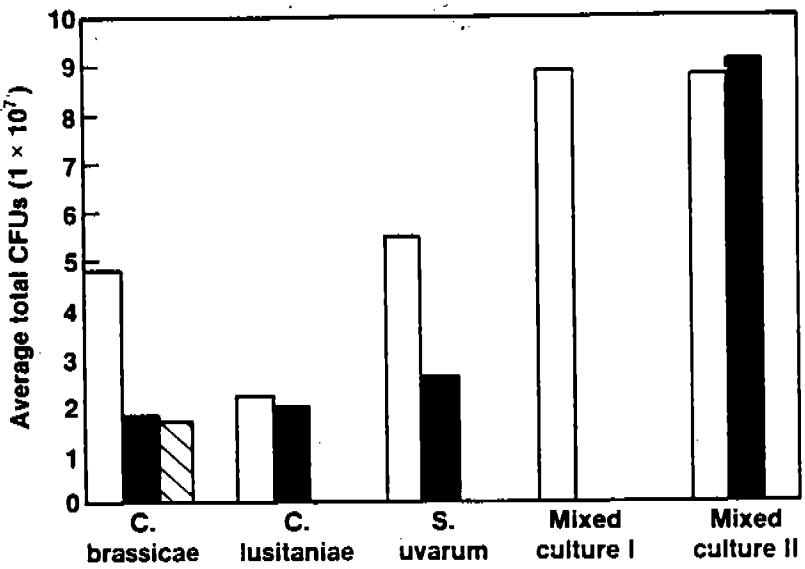


*Mixed Culture I- B. clausenii and S. cerevisiae
**Mixed Culture II- C. lusitaniae and S. uvarum
***Mixed Culture III- C. lusitaniae and C. brassicae

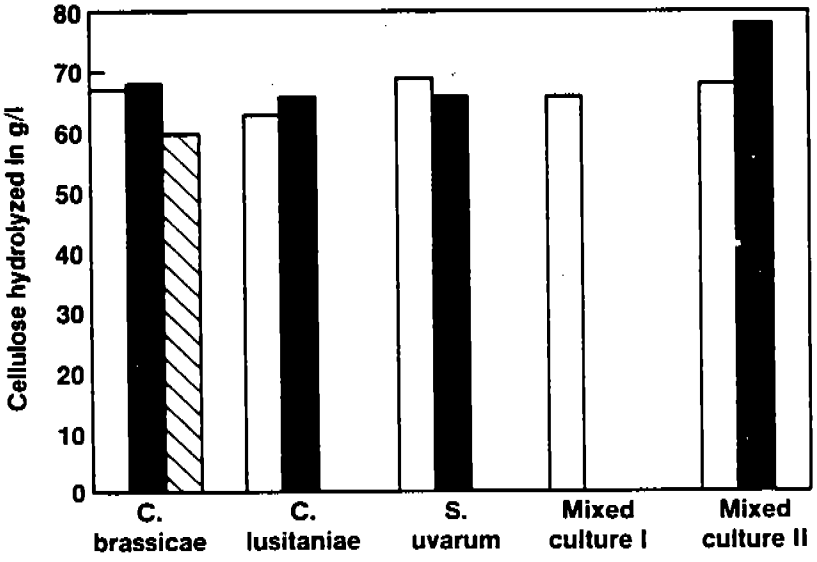
3. Comparison of mixed cultures at selected temperatures with 13 IU/g Genencor (batch I) cellulase enzyme loading at 10% Sigmacell-50 substrate concentrations for 2 days (□), 4 days (▨) and final day (■) percent equivalent conversions.



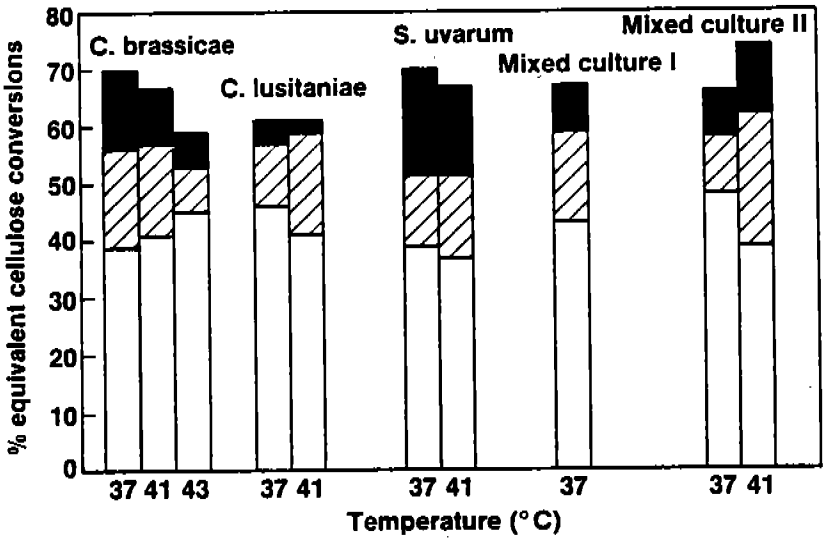
4. Comparison of average residual sugars for large scale SSFs at 37 (□), 41 (■) and 43°C (▨) over a seven day period and a Genencor (batch II) cellulase loading of 13 IU/g 10% Sigmacell-50 cellulose substrate.



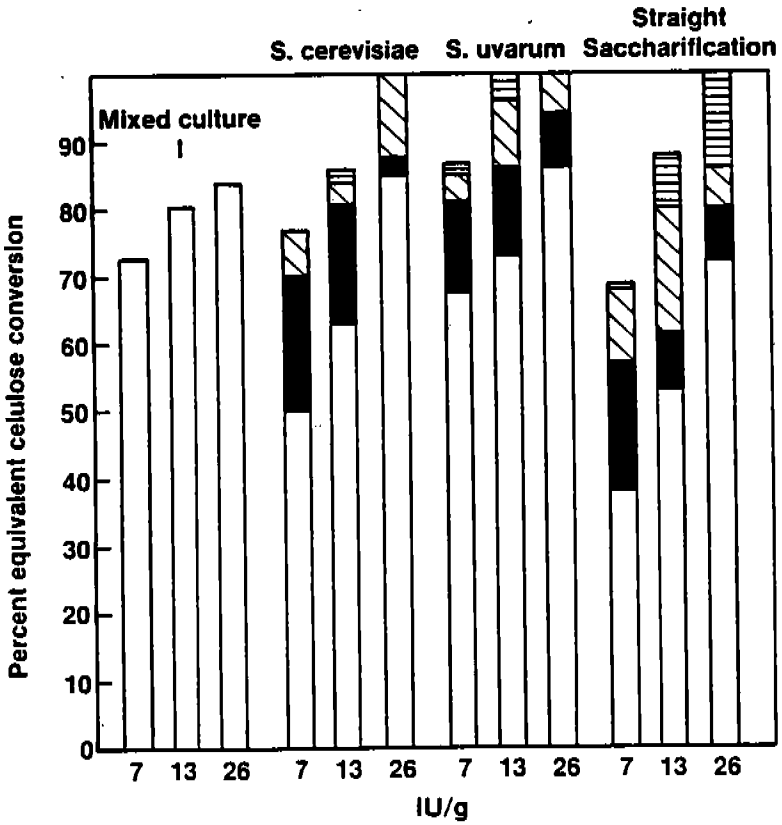
5. Average total colony forming units for large scale SSFs at 37 (□), 41 (■) and 43°C (▨) with a Genencor (batch II) cellulase loading of 13 IU/g 10% Sigmacell-50 cellulose substrate over a seven day period.



6. Cellulose hydrolyzed in g/l for large scale SSFs at 37 (□), 41 (■) and 43°C (▨) with a Genencor (batch II) cellulase loading of 13 IU/g 10% Sigmacell-50 cellulose substrate over a seven day period.



7. Percent equivalent cellulose conversions as a function of temperature for large scale SSFs with a Genencor (batch II) cellulase enzyme loading of 13 IU/g of 10% Sigmacell-50 substrate at 2 days (□), 4 days (▨) and final (■).



8. Percent equivalent cellulose conversions for straight saccharification and wheat straw SSFs as a function of Genencor cellulase (batch II) loadings of 7, 13 and 26 IU/g 7.5% glucan. β-glucosidase was supplemented at given ratios of 0:1 (□), 1:1 (■), 4:1 (▨) and 8:1 (⊟) parts of β-glucosidase activity per IU of Genencor at 37°C.